The Obligatory Intermediacy of a Cholesta-5,7-diene System in the Hepatic Biosynthesis of Cholesterol

By M. AKHTAR, D. C. WILTON and K. A. MUNDAY
Department of Physiology and Biochemistry, University of Southampton

(Received 8 August 1966)

The role of a cholesta-5,7-diene system in cholesterol biosynthesis has been considered in a number of publications in recent years (Dempsey, 1965; Dvornik, Kraml & Bagli, 1966). The present communication describes an approach based on a hydrogen isotope effect that establishes the obligatory nature of a cholesta-5,7-diene system (Scheme 1, reaction 3) in the biosynthesis of cholesterol.

During our studies on the mechanism of the Δ^7 -steroid-reductase-catalysed conversion of 7-dehydrocholesterol (Scheme 1, partial structure I, reaction 3) into cholesterol (Scheme 1, partial structure II, reaction 3), we have shown that the 7α - and 8β -hydrogen atoms of the cholesterol are derived from the 4-position of NADPH and a proton source respectively. When this conversion of structure (I) into structure (II) (Scheme 1, reaction 3) was carried out in the presence of tritiated water the cholesterol produced had radioactivity at both positions 7 and 8 (Wilton, Akhtar & Munday, 1966). The presence of tritium at the 7α -position was attributed to the presence of an enzyme system in rat liver that catalysed an equilibrium of hydrogen atoms between water and the 4-position of NADPH (Popják, Goodman, Cornforth, Cornforth & Ryhage,

1961) as suggested by Scheme 1, reaction (2), and the overall reaction as given in Scheme 1. Our previous work further suggested that in the conversion of 7-dehydrocholesterol into cholesterol the relative radioactivity associated with the formation of the 7α - and 8β -bonds was 3:1 (Wilton *et al.* 1966).

It follows from our previous results that, if a cholesta-5,7-diene system (Scheme 1, reaction 3) is an obligatory intermediate in cholesterol biosynthesis, then the conversion of a distant precursor into cholesterol in the presence of tritiated water should result in the incorporation of some radioactivity at positions 7 and 8. Further, the radioactivity at position 7 should be located entirely in the 7α -position and the relative activities between positions 7 and 8 should be 3:1.

For this study cholesterol was biosynthesized from squalene by using a 500 g. cell-free supernatant of rat liver in the presence of tritiated water under aerobic conditions. The resulting cholesterol was isolated and purified as previously described (Wilton *et al.* 1966; Akhtar & Marsh, 1966).

Although the biological conversion of squalene into cholesterol in the presence of tritiated water should result in the incorporation of tritium at positions 4, 7, 8, 9, 14, 24 and also possibly 25

Scheme 1.

Scheme 2.

(M. Akhtar, K. A. Munday & D. C. Wilton, unpublished work), the present communication refers only to positions 7 and 8.

The biosynthesized cholesterol (III) was degraded by the following sequence of reactions. A solution of cholesterol (2.8g.) and acetic anhydride (12ml.) was boiled under reflux for 2hr. After being cooled the reaction mixture was treated with acetic acid (36ml.), anhydrous sodium acetate (2g.) and chromium trioxide (3g.), and then stirred under an atmosphere of nitrogen for 5hr. at 50°. Addition of water (7ml.) gave, after filtration, 7-oxocholesterol acetate (Scheme 2, IV) in 30% yield. This was reduced with lithium aluminium hydride and the resulting diol benzoated to give the 3,7-dibenzoate (Scheme 2, V) (Fieser, Fieser & Chakravarti, 1949). In this conversion of cholesterol into the dibenzoate both hydrogen atoms at position 7 of the original cholesterol were removed and 15.0% of the radioactivity was lost (Table 1). If a cholesta-5,7-diene

system were an obligatory intermediate in the biosynthesis of cholesterol, the 15.0% of radioactivity lost should be entirely from the 7 α -position. This was confirmed by the conversion of cholesterol into the 5 α -hydroperoxide (Scheme 2, VII), when again 15% of the radioactivity was lost. It is well established that in the conversion of cholesterol into the 5 α -hydroperoxide (Scheme 2, VII) it is the 7 α -hydrogen that is stereospecifically lost (Nickon & Bagli, 1959).

The determination of radioactivity at position 8 proved more difficult and was eventually solved by the conversion of the dibenzoate (Scheme 2, V) into 7-dehydrocholesterol benzoate (Scheme 2, VI) (Haslewood, 1938; Fieser, 1953). This overall conversion of cholesterol into 7-dehydrocholesterol benzoate represents the removal of all hydrogen atoms from both the 7- and 8-positions and is accompanied by a 20.2% loss of radioactivity (Table 1). The net losses of radioactivity when the

Table. 1. *Biosynthesis of cholesterol from squalene in the presence of tritiated water and the distribution of label at positions 7 and 8*

Samples (10 ml.) of a 500 g. cell-free supernatant of rat-liver homogenates (1.25 ml. of 0.1 M-sodium phosphate buffer, pH 7.3/g. of liver) were incubated with 0.02 ml. of squalene at 37° for 3 hr. under oxygen. Each flask contained 30 μ moles of glucose 6-phosphate, 17 μ moles of glutathione, 5 μ moles of NADH and 2.5 μ moles of NADPH, and 200 mc of tritiated water. The isolated cholesterol from each flask had 150000 counts/min. The radioactive samples were dissolved in NE213 or NE220 liquid-scintillation fluid (Nuclear Enterprises Ltd., Sighthill, Edinburgh) and counted in an LDL 2022 liquid-scintillation counter (Isotope Developments Ltd., Beenham, Berks.) at 15% efficiency. All samples were corrected for quenching by internal standardization.

	Cholesterol (III)	5 α -Hydro- peroxide (VII)	7-Oxocholes- terol acetate (IV)	3,7-Diben- zoate (V)	7-Dehydro- cholesterol benzoate (VI)	% of total radioactivity	
						At C-7	At C-8
Counts/min./m-mole	38170	32300	32570	32380	30470	15.0	5.2
Loss of activity (%)	0	15.3	14.7	15.2	20.2		

hydrogen atoms are removed from positions 7 and 8 are therefore 15.0 and 5.2% respectively. This corresponds to a radioactivity ratio loss 2.9:1 associated with the 7 α - and 8 β -C-H bonds; this is in close agreement with our expected ratio 3:1 (see above).

These experiments, in our view, conclusively establish the obligatory role of a cholesta-5,7-diene system (Scheme 1, reaction 3) in cholesterol biosynthesis.

D. C. W. thanks the Science Research Council for a Research Studentship.

Akhtar, M. & Marsh, S. (1966). *Biochem. J.* (in the Press).
 Dempsey, M. E. (1965). *J. biol. Chem.* **240**, 4176.
 Dvornik, D., Kraml, M. & Bagli, J. F. (1966). *Biochemistry*, **5**, 1060.
 Fieser, L. F. (1953). *J. Amer. chem. Soc.* **75**, 4386.
 Fieser, L. F., Fieser, M. & Chakravarti, R. N. (1949). *J. Amer. chem. Soc.* **71**, 2226.
 Haslewood, G. A. D. (1938). *J. chem. Soc.*, p. 224.
 Nickon, A. & Bagli, J. F. (1959) *J. Amer. chem. Soc.* **81**, 6330.
 Popják, G., Goodman, de W. S., Cornforth, J. W., Cornforth, R. H. & Ryhage, R. (1961). *J. biol. Chem.* **236**, 1934.
 Wilton, D. C., Akhtar, M. & Munday, K. A. (1966). *Biochem. J.* **98**, 29c.

Biochem. J. (1966) **101**, 25c

A New Placental Metabolite of Oestr-4-ene-3,17-dione: a Possible Source of Error in Oestrogen Estimation

By JOHN D. TOWNSLEY and HARRY J. BRODIE

Worcester Foundation for Experimental Biology, Shrewsbury, Mass. 01545, U.S.A.

(Received 1 August 1966)

During studies on the mechanism of aromatization of C₁₈ and C₁₉ steroids Townsley, Possanza & Brodie (1966) have isolated a placental metabolite of 19-norandrostenedione (oestr-4-ene-3,17-dione) that has been identified by chromatographic, microchemical, stereochemical and isotope-dilution techniques as 1 β -hydroxy-19-norandrostenedione (1 β -hydroxyoestr-4-ene-3,17-dione). The production of this metabolite is of especial interest for three reasons. (1) Treatment of the compound with either

acid or base results in the formation of oestrone (3-hydroxyoestra-1,3,5(10)-trien-17-one). Consequently, any such material present in extracts of tissues or body fluids used for the estimation of oestrogens by the commonly used procedures (e.g. Brown, Bulbrook & Greenwood, 1957) would be converted into oestrone in this way and result in artificially elevated values. (2) 1 β -Hydroxylation was suggested as a possible intermediate step in the transformation of androgens into oestrogens by